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Differential Electrophoretic Separation of Cells
and its Effect on Cell Viability

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Abstract

This project was undertaken to apply an electrophoretic
separation method to the separation of cells. To determine
the efficiency of the separation, it was necessary to apply
existing methodology and develop new methods to assess the
characteristics and functions of the separated subpopulations.

Through appropriate application of the widely used iso-
electric focusing procedure, a reproducible separation method
was developed. Cells accumulate at defined pH and 70-80%
remain viable. The cells are suitable for further biologic,
biochemical and immunologic studies.

TABLE OF CONTENTS

Introduction

This project was undertaken for the development of a method for the preparative electrophoretic separation of viable cells. For cell populations consisting of some that differ significantly in morphological appearance, the evaluation of the success of the procedure is simple. A more challenging problem arises from the need to separate cells from populations like lymphocytes, that are morphologically similar in appearance but functionally heterogeneous. The importance of separating lymphocytes cannot be overstated. Besides their immediate role in defense against acute and chronic bacterial and viral diseases, their role in allergy, respiratory problems, autoimmune diseases and malignant diseases is being constantly probed. Concomitant with such an undertaking is the development of appropriate methods for examining the separated subpopulations so that specific functions, characteristics, and interactions might be better understood.

The history, background and applications of cell electrophoresis were presented in our initial proposal. It was also pointed out that because of the need for special and expensive instrumentation preparative electrophoretic separation of cells is not carried out in many laboratories. Most existing information comes from the use of the DeSaga Cell Separator, (1), (2), (3) and some from individually designed electrophoretic equipment (4), (5).

With the conviction that electrophoretic separations in low gravitational fields could be more efficient than at earth's gravity and impressed with the need to proceed rapidly, we focused our attention primarily on adapting the procedure to the simple, available, relatively inexpensive, widely used isoelectric focusing apparatus. The details of the procedure that evolved for obtaining a reproducible separation have been summarized and will be published. A prepublication copy is appended. In this report, we will extend the description of the parameters examined for electrophoresis and the methods used for characterizing the subpopulations of cells. Methods applied to define specific functional activities will be described, along with an evaluation of their advantages, limitations and suggested modifications which seem appropriate but have yet not been tried.

Procedures for maintenance of viable cells that are easily accomplished in the laboratory may not be suitable or easily carried out on a flight mission or in a space laboratory. Consideration of how cells can be maintained will be discussed including potential uses for separated cells that are not viable.

Method for Isoelectric focusing of peripheral leukocytes

Collection and preparation of cells

Leukocytes were removed from freshly drawn rabbit or human blood following sedimentation of erythrocytes with Dextran 150, according to the method described by Leise et al (6). When lymphocyte populations were desired, five ml of blood were layered over 3ml of Hypaque-Ficoll solution (H-F) prepared according to the recommendation of Boyum (7). The solution consisted of ten parts of Hypaque (33.9% Na diatrizoate), which is supplied as a sterile aqueous solution 50% ^{W}/v by Winthrop Laboratories, New York, N.Y. 10016 and 24 parts of 9% ^{W}/v Ficoll (Pharmacia Fine Chemicals, Piscataway, New Jersey 08854). The procedure was carried out in 12 x 100 or 150mm screw - capped siliconized tubes. Immediately after layering the blood on the H-F solution, the tubes were centrifuged at 400 x g, 24°C, for forty minutes. The lymphocyte layer was removed with a Pasteur pipette.

Cells obtained from either procedure were added to RPMI-1640 tissue culture medium containing 25mM Hepes buffer obtained from Grand Island Biological Co. Grand Island, New York, 10472. The suspended cells were centrifuged at 400g for 10 minutes and the supernatants removed. This washing of the cells was repeated two additional times. The cells were suspended in exactly 1.0ml of RPMI and counted. Immediately before addition to the column, the cell suspension was centrifuged and the supernatant removed. The pelleted cells were suspensed in a small volume of the midpoint gradient solution and introduced into the previously prepared column.

Medium for electrophoretic separation

Unlike conventional electrophoresis, for which an isotonic buffer of physiologic pH is required, the medium for isoelectric focusing must consist of a non-ionic density gradient solution and an appropriate Ampholine or mixture of polyaminopolycarboxylic acids. Salts must be excluded or kept to a minimum. Since sucrose is the least expensive and most widely used substance for establishing the density gradient necessary to stabilize the pH gradient, it was tried first. After isoelectric focusing a separation of cells was accomplished but the cells were all in very poor condition. The effect of a Ficoll density gradient on cells was much less destructive. Cells remained viable but the viscosity of the denser solutions at low temperatures was so great that separation was poor and the collection slow and difficult.

Dextran 40, Lot No. 8334 Nutritional Biochemical Corp., (Now ICN Corp.), Cleveland, Ohio 44128, a product used in our laboratory for the separation of cells by discontinuous density gradient centrifugation, provided the most satisfactory and effective separation. Since dextrans are heterodisperse in molecular weight and batches vary widely, their effect on cell viability and on distribution, both for gradient separation and for isoelectric focusing, must be previously established.

Ampholines are provided as 40% W/v solutions by the LKB company, Rockville, Md. It is possible to obtain mixtures that will provide pH density gradients as broad as 3-10 and as narrow as 2.5-4. Since the product is expensive and ever-increasing in price, we felt it was also necessary to establish the minimum concentration at which an effective separation could be accomplished. Although it is not a final and definitive appraisal, because that would necessitate equating the Ampholine requirement with each sample size or number of cells introduced into the column, good separations can be obtained with as little as 1 ml of the 40% solution. Our recommendation for the use of 2.0 ml for the 110 ml column was made because that amount would be effective for separating a broader range of cell concentrations.

Preparation of column and introduction of gradients and sample

Columns were washed thoroughly in R.B.S. 25 detergent (Fisher Scientific Co.) and thoroughly rinsed. Any detergent known not to interfere with cell viability and subsequent cell culture would also serve. The columns were siliconized with Siliclad (Clay Adams). Despite this effort a variable number of cells was always lost by adherence to the column walls. Other agents were tried but with no greater success.

Sterilization of the column was carried out by rinsing thoroughly in 70% ethyl alcohol and drying in an oven at 56°C. If an ethylene oxide sterilizer, like the Castle Powerclave, is available and is functioning properly, so that there is no residue left on the glass, it provides a very suitable sterilization procedure. The column withstands autoclaving at 200°F for 40 minutes but the plastic that encloses and protects the electrodes becomes discolored. Therefore, carrying out this method of sterilization on a daily basis is not recommended. To prevent contamination during electrophoresis the entrance and exit channels of the column were covered with gauze and secured with adhesive tape.

The gradient may be introduced manually as a discontinuous gradient, but the probability of contamination is enhanced. Adding sterile solutions to a previously sterilized mechanical gradient

device is definitely preferable. In either case, dense and light
gradient solutions are required. The dense gradient solution
consisted of 28.0 gm Dextran 40, 2.0 ml Ampholine (40%) and 70 ml
distilled water. For lymphocytes, Ampholine 5-7 was found to be
superior to Ampholine 4-6 or 3-10. The light gradient solution
consisted of 0.5 ml of Ampholine and 60 ml of distilled water.
In practice, a cathode solution consisting of 0.4 ml mono-
ethanolamine and 10 gm Dextran diluted to a final volume of 20 ml
was introduced manually into the column first, followed by the
gradient itself which was mechanically introduced. When the
column was half filled, it was interrupted and the sedimented cells
were suspended in some of the gradient solution, introduced into
the column with a sterile syringe, and the filling of the column
with gradient solution continued. The upper electrode solution
which consisted of 0.1 ml H_3PO_4 (85%) and 9.9 ml of distilled was
added manually until it just covered the platinum wire at the top
of the column.

To all solutions, Penicillin-Streptomycin Solution (Grand
Island Biological Company) was added and distributed amongst the
four solutions so that there were 250 units of penicillin and
250 ug of streptomycin per ml..

Temperature

The temperature was controlled by pumping refrigerated water
through the outer and inner cooling jackets. When the column is
maintained at 5^oC, the viscosity of the dense solutions increases
to the point where subsequent collection becomes slow and difficult.
Rather than change the temperature at the end of the run and dis-
tort the fractions during the collection period, we found 7^oC to
be adequate for the electrophoresis and collection periods. For
some cells this may not be optimal and other temperatures should
be tried.

Voltage and Current

The voltage was set initially so that the current registered
4-4.5 mA. Increases in voltage were made at frequent intervals
so that the current was maintained at the starting level. After
2-3 hours the voltage was set at its maximum (1200V). The current
then fell gradually. In 24-48 hours, when the pH gradient is es-
tablished. The current becomes stabilized around 1-1.5 mA. No
further separation occurs after this. The electrophoresis is dis-
continued and the column is then emptied.

Collection of Cell subpopulations following electrophoresis

For the collection of electrophoretically separated macro-molecules, the fractions should be about 0.5 ml each. From the appearance of the cell layers on the column, distances of 1 cm or 4 ml seemed more appropriate. Therefore 4 ml fractions were collected as rapidly as possible. Pressure was applied at the top opening of the column with a peristaltic pump. Precautions were taken during collection to maintain sterility.

Characterization of cell subpopulations following isoelectric focusing

pH-at which cells accumulate

At the periphery of the column, the pH represents that of the electrode solutions. Cells that come off in the first tubes and the last are relatively few in number, usually nonviable and could probably represent artefacts of collection. By the third or fourth tube the pH represents that of the true Ampholine gradient. Sub-sequent tubes show smooth and regular decrements in pH, until the anode solution is reached. When cells are obtained from very alkaline fractions, they appear fragmented and distorted. Between pH 6.8-7.0 there is a definite increase in the number of poly-morphonuclear leukocytes and eosinophiles, when the cells ap-plied consist of all the peripheral leukocytes obtained from a Dextran-150 separation. If the cells are obtained from the upper layer of a Hypaque-Ficoll separation, the cells are predominantly lymphocytes and few cells are obtained in alkaline fractions.

The distribution of peripheral lymphocytes according to pH may be very significant. A summary of this distribution, observed in cells from six rabbits, may be seen in table 1 in the appended publication. Since lymphocytes vary in size, age and function, a profile of their pH distribution is easily obtained and might in itself, be very meaningful in understanding the immune response of different individuals.

Density Gradient

Just as the density gradient stabilizes the pH gradient, it is conceivable that it also stabilizes the cell separation and prevents heavier cells from falling through the medium. This may be of no significance in lower gravity. It is, however, very clear that sucrose in high concentrations is destructive to cells and that dextrans provide a protective influence. Cells exposed to appropriate dextrans are definitely more resistant to lysis.

The use of dextran could be responsible for their survival. Addition of dextrans to the medium in which conventional electrophoresis of leukocytes is being carried out could serve to support and protect the cells.

The actual density or specific gravity of each fraction was determined from a standard curve after obtaining the refractive index on an Abbe refractometer.

Morphologic characteristics of separated cells as determined by light microscopy

Trypan Blue Staining

When a drop of sedimented cells is placed in a drop of trypan blue stain (about .04%) made up in normal saline, on a small area of a slide, defined by a paraffin ring and covered with a cover slip, the cells can be observed up to a magnification of a thousand. If the procedure is destructive to the cells or if there are more dead cells in some subpopulations than in others, the cells that permit entrance of dye into the cell and take on a blue stain is obviously increased. Whether the non-staining cells are truly viable remains controversial among some investigators. In conjunction with other parameters for the assessment of viability, we find it a very effective, simple means of differentiating those cells that exclude dye from those that permit its entrance into the cell.

If cells are counted using trypan blue solution as diluent, an immediate estimate of the relative numbers of stained and unstained cells in each fraction can be made.

Wright Stain

A drop of cell suspension was spread evenly on a slide, previously prepared with a thin film of Mayers albumin fixative. The cells were air dried and stained with Camco Quick Stain (Scientific Products), a buffered Wright's stain.

Distortion is frequent on drying, when cells are separated from blood or serum. Drying with an additional drop of albumin solution or fibrinogen or gelatin solution was tried but did result in any further improvement. The color and appearance of similarly stained cells from different parts of the column varies even with lymphocyte preparations. Between pH 6 and 7 cells appear bluer with only an occasional pink staining cell in these fractions. In the fractions more acid than pH6, cells do not stain as intensely

blue. Larger mononuclear cells predominate. Cells from the very acid fractions, exposed to the anode phosphoric acid solution appears as very large mononuclear cells with large amounts of lighter staining, slightly granular cytoplasm. Cell particulates from disrupted cells also seem to accumulate in the acid region. It is possible that ampholyte binding to cell surface has occurred.

Fluorescent Stain

Fluorescent staining of rabbit lymphocytes to determine the kind and amount of immunoglobulins on their surface was carried out by either of two methods. A drop of cell suspension was air-dried as previously described and covered with fluorescein conjugated goat antirabbit IgG (Microbiological Associates, Bethesda, Md.). The slides were maintained at ambient temperature in a moist chamber for 2 hours. They were washed repeatedly in saline, and finally in water and allowed to dry again. A drop of buffered glycerol mounting medium was placed on the slide and spread evenly over the surface when a large cover slip was superimposed on the slide. Absence of bubbles is essential. The slides were observed immediately using a Bausch & Lomb microscope with a fluorescence attachment.

To insure removal of non-specific fluorescent material, not actively bound to cells, 0.5×10^6 suspended cells were washed repeatedly with Hanks' balanced salt solution plus 0.5% Dextran 40. Two ml of a 1/10 dilution of the fluorescein conjugated goat anti-rabbit IgG solution were added to the washed sedimented cells. The suspension was agitated gently for two hours at ambient temperature. Centrifugation followed by removal of the supernatant was repeated until a drop of dried supernatant, showed no fluorescence. The cells were placed on prepared slides, as previously described, dried, covered with glycerol solution and examined. These preparations were superior.

Cells from the lower part of the column appeared to have a larger number of cells with uniformly distributed immunoglobulin. The greatest localization occurred around pH 6.0, followed by some fractions with no fluorescent stained cells and then again some uniformly stained cells in the pH 5.0 fraction. No immunoglobulin binding to cell surfaces was observed in cells from fractions more acid than pH 5.0. A judgment about labeling these "B" cells must wait for more definitive studies.

There were many lymphocytes that exhibited polarized or "capped" fluorescent staining in the cells examined, but uniform staining predominated.

Biologic & Biochemical Characterization
of Separated Cells

Growth on culture

Growth on culture could conceivably be considered the ultimate parameter for assessing cell viability. Unfortunately, the poly-morphonuclear leukocyte is an end cell and will not undergo further divisions. Some lymphocytes will replicate and some will not. This attempt at cell culture is therefore more effective in localizing those cells capable of replication. Failure to replicate does not imply cell death. Some cells have remained viable on culture for the 72 hour period over which we have examined them, without in-creasing in number. Cells from some fractions close to pH 4.0, exhibit good replication on culture in RPMI-1640. Cells from alka-line fractions are either not viable or die off during this period. This procedure was described and the results shown graphically in the appended publication. (See Figure 7)

Oxygen uptake

Using a YSI O_2 electrode connected to a recorder, it was possible, when the cell population or subpopulation was large enough (1 x 10^7 cells), to measure O_2 utilization, at 37°C, of cells suspended in a standard incubation medium. (8). Differences in O_2 uptake were observed in lymphocytes obtained from different fractions fol-lowing isoelectric focusing. Mixed peripheral lymphocytes, ob-tained from a Hypaque-Ficoll separation, were compared with cells from spleen, thymus and lung teased from a rabbit which was sacrificed. Some cells showed greater O_2 utilization after iso-electric focusing than exhibited by the mixed population obtained from the Hypaque-Ficoll gradient prior to electrophoreses.

Cell Suspensions	Oxygen $\mu l/10^6$ cells/hr.
Peripheral (H-F)Lymphocytes	0.07 μl
Splenic Lymphocytes	0.78
Thymic Lymphocytes	0.44
Lung macrophages	3.60

After isoelectric focusing	
*pH 6-11 lymphocytes	1.30
pH 4.5-5.5	0.50
pH 1.6-4.5	1.30

*Pool of the cells from these fractions

This supports the viability observations made with trypan blue exclusion.

Incorporation of labeled precursors

^{14}C-leucine

Uptake of an amino acid could result from binding or incorporation into newly synthesized <u>protein</u> and is another method for assessing cellular activity. <u>Cells may</u> not replicate, but if they survive on culture, cell substance and enzyme replacement must occur and will be reflected ir ^{14}C-leucine incorporation into cell proteins. Cells, 0.5 x 10^6, were suspended in RPMI-1640 containing 1 uCi ^{14}C-leucine for 4, 24 or 48 hours. The cells were pelleted by centrifugation, washed three times with Hanks' balanced salt solution plus 0.5% Dextran 40. The suspensions were centrifuged again and 2 ml of 5% trichloroacetic acid were added to precipitate the proteins. Precipitates were washed twice with cold trichloroacetic acid, twice with methanol, air dried, dissolved in 0.1 N NaOH, transferred to counting vials containing Beckman's Ready-Solv #6 scintillation fluid and counted in a Beckman Liquid Scintillation Counter. Controls consisted of subjecting aliquots of the same numbers of cells at $56^\circ C$ for 30 minutes to the same procedure.

When 0.5 x 10^6 cells were not available, from each fraction, following focusing, to be consistent, the sample size was reduced, so that incorporation by the same number of cells could be compared.

Lymphocytes obtained directly from a density gradient separation show active incorporation in four hours with increases of 23-87 times that level of incorporation in 24 hours.

Following isoelectric focusing, uptake of ^{14}C-leucine in four hours is definitely lower and does not rise at the same rate in 24 hours as unfocused cells.

Attempts made to stimulate or revitalize focused cells, to bring them back to a comparable level of activity, included additions of adenine triphosphate to the medium, cyclic adenine mononucleotide, insulin, glucagon, estradiol and progesterone. No remarkable changes were noted. Since so many of these responses are concentration dependent, much more work in this area is required. The most successful effort consisted of washing the cells repeatedly in a basal isotonic salt medium. The standard incubation medium used for O_2 uptake studies proved to be most effective. The cells were suspended and washed before the addition of medium, containing luCi ^{14}C-leucine per ml. Despite some improvement, to obtain counts of any reasonable level, 24-48 hours of incubation was necessary.

In one experiment, subsequently paralleled by others, cells from pH 5.8 showed better incorporation of labeled leucine than those accumulations immediately surrounding them. Good activity was demonstrated by cells from pH 5.3 (247 DPM/10^5), maximum incorporation was demonstrated by cells from pH 5.2 (3297 DPM/10^5). Gradually decreasing incorporation was demonstrated by cells from the upper more acid fractions from the column.

Many factors could account for the lower activity of cells following isoelectric focusing. Changes in activity of separated cells could result from the loss of impact of one cell on other cells. It might also result from loss of the effects of products from other cells, the effects of the long period of exposure to cold ($7^{\circ}C$), the effects of the electrophoretic procedure per se or the effects of the low ionic strength medium. This could be a most productive area of research in distinguishing the biochemical activity and potential of separated subpopulations of cells.

Protein and enzyme concentrations of separated subpopulations

The determination of protein and enzyme concentrations of the populations of cells collected after an electrophoresis can serve many functions. Primarily, it may serve to characterize the cells or help differentiate one type from another. Since very small amounts of material are required for most enzyme analyses, there is no problem in obtaining sufficient numbers of cells. The methods are simple and reproducible. Since the samples require lysis prior to analysis, the problem of maintaining cells in viable condition until the termination of a mission is removed.

The established procedure in our laboratory is to collect cells separated by either the density gradient procedure or isoelectric focusing procedure previously described and add the washed, counted cells directly to glycerine-non-idet solution (0.5% non-idet P-40 (Shell) dissolved in 50% glycerine). Cells lysed in this manner can be kept in the freezer at $-20^{\circ}C$. for up to two years with no loss in some enzymes and inappreciable loss in others.

We examined protein and lactate dehydrogenase (LDH) concentrations of lymphocytes, separated by isoelectric focusing, and compared these concentrations with that of lymphocytes not subjected to electrophoresis and with lymphocytes separated by density gradient centrifugation.

We do not have sufficient data to make any conclusive statements, other than that the assays are valuable in differentiating the activity of cells from the various fractions obtained following isoelectric focusing. We can point out, however, from the few

experiments carried out that there is a reduction in LDH and protein concentrations in cells following the isoelectric focusing procedure. The cells with the lowest levels correlate well with our appraisal of their nonviability as determined from their morphological appearance. Frayed, vacuolated pale staining cells have low protein and LDH levels irrespective of the pH at which they have accumulated. The levels of cell protein and enzymes are known to vary in different individuals. Therefore, at this time, comparison must be restricted to the cells from each individual separation procedure.

The following information has been taken from two different separations. The fractions compared appeared to be morphologically intact. The LDH and protein concentrations and specific activities are tabulated. Many more fractions have been analyzed in these and other experiments, but not enough to summarize in any definitive manner.

	pH of fraction	$LDH/\mu/10^6$	Protein $\mu g/10^6$	Specific activity
Experiment 1	5.9	72	22	3.3
	5.55	83	37	2.2
	5.5	89	45	2.0
	5.3	54	23	2.3
	5.0	48	22	2.2
	6.6	112	84	1.3
Experiment 2	5.9	28	26	1.1
	5.62	33	20	1.7
	5.2	28	22	1.3
	5.08	39	12	3.3

Because of the fragility of cells and possible leakage of cell proteins into the medium, the supernatant portion of each fraction from the column, obtained after the sedimentation of the cells, was analyzed for protein and LDH. All were negative for protein. The more sensitive LDH determination indicated only two fractions, one pH 6.5 and one pH 5.78 that had any measurable LDH concentrations.

The former was 24 units in the total supernatant and the latter
16 units. This is a negligible amount. The slightly lower LDH
values, observed in cells following electrophoresis, could arise
as a result of their decreased activity - possibly due to low
temperature exposure.

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Immunologic characteristics of
separated subpopulations of lymphocytes

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To understand the functional diversity of a morphologically
similar population of cells requires quantitative, objective
methodology. To stimulate some cells and increase the number of
specifically committed cells rabbits were hyperimmunized with
three types of antigens. Some were injected with suspensions of
sheep erythrocytes (SRBC). This antigen was once believed to
stimulate a B cell response exclusively and is now believed to
stimulate both B & T cells. Purified protein derivative of
Mycobacterium tuberculosis (PPD) was administered to other rabbits
to stimulate a T cell response. A third group received injections
of human serum albumin (HSA) because it offered the opportunity
to study lymphocyte surface binding capacity before and after
immunization.

1. Hyperimmunization with SRBC.

At the time of peak antibody production, there was an in-
crease in the number of cells that accumulated in the more
acid part of the isoelectric focusing column.

When the separated cell subpopulations were examined for
cells possessing antibody, capable of lysing sheep erythrocytes
in the presence of complement, using the Jerne plaque technique,
quantitation was difficult. Cells with lytic capacity were
observed in fractions from pH 5.8 and pH 5.5. No judgment
could be made about the extent of the increase in the number
of committed cells in hyperimmunized animals.

We obtained a quantitative evaluation using the following
method. Cells ($.25 \times 10^6$) from each fraction were washed and
suspended in veronal buffered saline with calcium and magnesium
(Complement diluent) so that the final volume was 1.05 ml.
Before the final volume was adjusted, 0.3 ml of SRBC suspension
(O.D. 540 nm was adjusted to 0.194 when hemolysis was complete)

was added, and 0.1 ml guinea pig complement diluted 1/20. This
suspension was incubated at 37°C for 30 minutes, followed by
centrifugation at 1400 rpm for 10 minutes. The clear super-
nates were removed and the degree of hemolysis of each one was
quantitatively determined from the optical density. Cells from
normal rabbits showed some slight lytic capacity. This was
more prominent at pH 5.5, and absent from cells obtained from
other fractions. From the hyperimmunized rabbits, the concen-
tration of lymphocytes capable of lysing sheep erythrocytes was
greatest in the populations at pH 6.7 (this increased on culture),
very high in cells from pH 5.5-5.7, and negative in cells from
fractions more acid than pH 4.5.

2. Immunization with PPD.

Forty eight hours after an intradermal injection of 5 t.u.
PPD, lymphocytes were removed from a rabbit that had been ex-
amined repeatedly during a preimmunization control period.
The distribution profile showed an increased accumulation of
cells at pH 5.2.

> % of total cells that accumulate at pH 5.2
> following isoelectric focusing
>
> Preimmunization   2.1% of total
>
> 48 hrs after 1st injection   8.5% of total
>
> 48 hrs after 2nd injection   11.3% of total *
>
> * This was the peak accumulation for this population
>   of cells.

No significant changes occurred in other fractions.

The hypothesis that these cells should be more responsive
to stimulation by a mitogen and by PPD was difficult to support.
The incorporation of tritiated thymidine into cultured lym-
phocytes requires at least 0.5×10^6 cells. We made the de-
cision to pool fractions so that replicate determinations
could be carried out.

Using our standardized procedure, of 72 hrs of culture,
followed by 18 hrs of exposure to 1 uCi of ^3H-thymidine, in a

total volume of 2.0 ml of RPMI-1640, with 5% fetal calf serum, we could demonstrate no specific increases in incorporation by cells cultured with PPD. Modifications in treatment of cells to offset the effects of cold exposure must be undertaken. Optimal concentrations of PPD must also be determined. The effects of mitogens like phytohemagglutinin, pokeweed and concanavalin A must also be explored further in efforts to evaluate the responses of separated cells.

3. Immunization with human serum albumin (HSA)

HSA was selected as another appropriate antigen because antibodies on lymphocyte surfaces might be detectable simply and quantitatively by measuring differences in capacity to bind to ^{125}I-HSA. This is a relatively inexpensive product which is commercially available (E.R. Squibb, New Brunswick, N.J.).

All rabbit cells bound ^{125}I-HSA firmly to some degree irrespective of the previous treatment of the rabbit. In vitro addition of phytohemagglutin to suspended cells, modified their ^{125}I-HSA binding capacity, except for cells from the pH 5.6 fraction. Cells from this fraction, obtained from previously HSA hyperimmunized rabbits, exhibited a higher in vitro binding capacity than any other cell population and were unaffected by PHA. These cells were 420% higher in binding capacity than PHA treated cells of a comparable fraction from a non-immunized control rabbit. This difference suggests a localization of specifically committed cells at pH 5.6.

Conclusion

A reproducible separation of peripheral leukocytes and lymphocytes by electrophoresis using suitable modifications of the isoelectric focusing method has been accomplished. Progress has been made in assessing functional characteristics of the separated cells. Much more research is required to identify the specific commitments of separated subpopulations and develop methodology suitably modified for quantitatively evaluating these various immunologic activities.

The importance of both the separation procedure and identification of the specific roles of the separated subpopulations cannot be overemphasized. Simple identification as a B or a T cell by a morphological technique is inadequate and an oversimplification. Understanding the many and varied roles of the cells involved in the immune response, as well as other types of cells, eventually can provide specific clones capable of synthesizing specific enzymes, mediators, hormones, antibodies and other important and sought after cell factors.

With current thinking on the value of replacement of lymphocytes in immunodeficiency diseases (9) and the involvement of the immune system in malignant diseases (10), autoimmune diseases, aging and allergy as well as infectious diseases, it would seem that separation of lymphocytes continues to be a valuable area for further research.

On a flight mission, the maintenance of cells in viable condition could pose a problem. Since freezing is l_thal to most cells, unless the rate of temperature decline is controlled. Usually, this is $1-3^{\circ}C/Min$, down to $-196^{\circ}C$, with subsequent storage in liquid nitrogen (11). It would seem that since dextran appeared to influence the stabilization of the cell surface in our separation procedure, its incorporation into the electrophoretic medium and culture medium would be a desirable addition.

As a peripheral benefit of this research, it must be pointed out that electrophoresis appears to provide a simple method for obtaining an immunoprofile, or a procedure for obtaining a quantitative distribution of the cells involved in the immune response. This should find useful clinical application.

Finally, we enjoyed working on this project and wish to thank many interested NASA scientists for their cooperation, advice and encouragement.

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